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Tepidimonas taiwanensis sp. nov., a novel alkaline-protease-producing bacterium isolated from a hot spring

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Abstract The bacterial strain designated I1-1^T was isolated from a hot spring located in the Pingtung area, southern Taiwan. Cells of this organism were Gram reaction negative rods, motile by a single polar flagellum. Optimum conditions for growth were 55°C and pH 7. Strain I1-1^T grew well in lower nutrient media such as 5–10% Luria–Bertani broth, and its extracellular products expressed alkaline protease activity. The *16S rRNA* gene sequence analysis indicates that strain I1-1^T is a member of β-Proteobacteria. On the basis of a phylogenetic analysis of 16S rDNA sequences, DNA–DNA similarity data, whole-cell protein analysis, physiological and biochemical characteristics, as well as fatty acid compositions, the organism belonged to the genus *Tepidimonas* and represented a novel species within this genus. The predominant cellular fatty acids of strain I1-1^T were 16:0 (about 41%), 18:1 ω7c (about 13%), and summed feature 3 [16:1 ω7c or 15:0 iso 2OH or both (about 26%)]. Its DNA base ratio was 68.1 mol%. We propose to classify strain I1-1^T (=BCRC 17406^T=LMG 22826^T) as *Tepidimonas taiwanensis* sp. nov.

Keywords *Tepidimonas taiwanensis* · β-Proteobacteria · Hot spring · Alkaline protease · Taxonomy

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Introduction

Proteases constitute one of the most important groups of enzymes, which are used in different industries in the detergent, food, pharmaceutical, leather, and silk (Gupta et al. 2002; Rao et al. 1998). Alkaline proteases are particularly important because they are both stable and active under harsh conditions, such as temperatures ranging from 50 to 60°C, high pH, and the presence of surfactants or oxidizing agents (Gupta et al. 2002; Banik and Prakesh 2004). Of late, organisms associated with hot springs in geothermal areas have received considerable interest. Recently, interest has been shown in the diversity of enzymes from thermophilic bacteria and their biotechnological potential (Niehaus et al. 1999; van den Burg 2003). Due to the extensive industrial importance of alkaline proteases, there is ongoing interest in the isolation of effective thermophilic bacteria strains that are capable of producing alkaline protease suitable for industrial applications.

In the course of the ongoing study of exploring the bacterial diversity of hot springs located in southern Taiwan, we characterized a novel species that had strong alkaline protease activity and did not appear to correspond to any recognized species in the genus *Tepidimonas*. The important characteristics of this isolate were compared with its nearest neighbors.

Materials and methods

Isolation and growth conditions

In May 2004, a sample (1,000 ml) of water was collected from a Sih-Chong-Si hot spring located in Pingtung county in southern Taiwan. The temperature of the spring water ranged from 55 to 60°C and the pH was approximately 8. The water samples were diluted with sterile distilled water, spread onto 10% Luria–Bertani (LB) agar plates (1.0 g of tryptone, 0.5 g of yeast

extract, 1.0 g of NaCl, and 15 g of agar (l^{-1})), and incubated at 55°C. The skim milk medium (10 g of skim milk and 20 g of agar (l^{-1})) was used to assess protease production, which was evidenced by the development of a clear halo zone-surrounding the bacterial colonies. The novel bacterial strain thus recovered was designated I1-1^T with alkaline protease activity.

Morphological and physiological tests

Strain I1-1^T was grown on 10% LB medium for 48 h at 55°C. The cell morphology was observed under a light microscope (Zeiss Axioskop, Jena, Germany) and the motility of exponential-phase cells was examined by the hanging drop method. Flagella staining was performed using Spot Test Flagella Stain (Difco); Gram staining was performed using the Gram Stain Set S (Difco, NJ, USA). Poly-β-hydroxybutyrate granule accumulation was observed under light microscopy after being stained with Sudan black (Fahy and Persley 1983).

The pH range for growth was determined by measuring optical densities (wavelength 595 nm) of the culture grown in 10% LB medium, whose pH was adjusted to values between 3 and 11 with appropriate biological buffers (Chung et al. 1997). To determine growth-temperature ranges, cells were incubated in 10% LB medium at temperatures between 15 and 70°C, while growth was determined by measuring the optical density of the culture with respect to time. Tolerance to NaCl was determined by adjusting the salinity of 10% LB medium to values between 0 and 3% NaCl. Anaerobic cultivation was performed on 10% LB medium under the Oxoid AnaeroGen system. Growth was considered to have occurred when the observed OD_{595} value exceeded twice the initial value (initial OD_{595} value after inoculation was 0.05) after 120 h incubation. Uninoculated control media had not shown increased OD_{595} value after 120 h incubation.

16S rDNA sequencing and phylogenetic analysis

Amplification and sequence analysis of the *16S rRNA* gene was performed as described elsewhere (Chen et al. 2001). Almost a full-length (1430 bp) sequence of strain I1-1^T was obtained and compared with other eubacterial sequences available from GenBank and Ribosomal Database Project II. The multiple-sequence alignment of strain I1-1^T and its closest relatives was performed using BioEdit software (Hall 1999). The phylogenetic reconstruction was inferred by the neighbor-joining method (Saitou and Nei 1987). A bootstrap analysis (confidence values estimated from 1,000 replications of each sequence) was performed by CLUSTAL w 1.7 program (Thompson et al. 1997). A phylogenetic tree was drawn using the TREEVIEW program (Page 1996). Sequence identities were calculated using the BioEdit software (Hall 1999).

Determination of the DNA base composition

The DNA sample was prepared as described by Pitcher et al. (1989) and degraded enzymatically into nucleosides as described by Mesbah et al. (1989). The obtained nucleoside mixture was separated by high-performance liquid chromatography using a Waters Symmetry Shield C8 column thermostated at 37°C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5% acetonitrile. Nonmethylated lambda phage DNA (Sigma, MO, USA) was used as the calibration reference.

DNA–DNA hybridizations

DNA–DNA hybridizations were performed with photobiotin-labeled probes as described by Ezaki et al. (1989). The hybridization temperature was 50°C and the reaction was carried out in 50% formamide. Each value obtained was the mean of two hybridization experiments.

Fatty acid methyl ester analysis

Cells were grown on Tryptic Soy Agar (TSA) (Difco) at 55°C. Cellular fatty acids were analyzed as methyl esters by gas chromatograph according to the instructions of Microbial Identification System (MIDI; Microbial ID) (Sasser 1990).

Analysis of protein electrophoresis patterns

Preparation of cellular protein extracts and SDS-PAGE analysis were performed as described by Pot et al. (1994). Numerical analysis was performed using AAB 1-D Advanced Software package (Advance American Biotechnology). The similarity between strains was expressed by the Pearson product–moment correlation coefficient (Pearson 1926) for both the position and the intensity of bands measured, and was converted for convenience to a percentage value.

Staining (zymogram) for protease activity was carried out according to the methods of Secades and Guijarro (1999), with slight modifications. The 10 μg of concentrated extracellular proteins was dissolved in the sample buffer without 2-mercaptoethanol (Laemmli 1970). Electrophoresis of proteins was conducted in an 8% (w/v) polyacrylamide gel, which copolymerized with 1% (w/v) sodium caseinate, using a Bio-Rad Mini Protein III apparatus at a constant voltage of 140 V for 2 h at 4°C. After electrophoresis, SDS was removed from the gel by soaking in 20% (v/v) isopropanol in 10 mM acetate buffer (pH 5.0) for 10 min, and then equilibrated in 50 mM Tris buffer (pH 8.0) for 15 min, and incubated at 55°C for 4 h. Finally, the gel was stained with Coomassie brilliant blue G-250 and destained, and the clear zones indicated the presence of protease activity.

Biochemical analysis

Strain I1-1^T was biochemically characterized using API 20NE and API ZYM (API bioMérieux) microtest systems according to the manufacturer's instructions at the testing temperature of 55°C. Biolog GNII microtiter test plates were used to determine carbon substrate metabolism, for which early log phase cultures were used as inoculum (150 µl well^{-1}). The plates were incubated at 55°C and examined after 24 and 48 h to allow the development of a purple color indicative of substrate oxidation.

Antimicrobial susceptibility testing

The susceptibility to antimicrobial agents was determined by the disk-diffusion method. The density of the bacterial suspension from exponential-phase culture was adjusted with sterile saline to the turbidity corresponding to 0.5 of the McFarland standard and then spread onto 10% LB medium and incubated at 55°C. Antimicrobial disks used (Dispens-O-Susceptibility Test Disks, Difco) included ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), rifampin (5 µg), penicillin G (10 µg), streptomycin (10 µg), and tetracycline (30 µg). Susceptibility was defined as zones of >3 mm, resistance as zones of <1 mm, and moderate resistance as zones of 1–3 mm to the edge of a disk.

Results and discussion

Phenotypic and morphological characteristics

Strain I1-1^T was isolated from water of a hot spring located in southern Taiwan. The bacterium grew well aerobically in lower nutrient media such as 5–10% of LB medium. The growth was poor in the normal concentrations of common complex media, such as Tryptic Soy, Nutrient, and LB medium.

Strain I1-1^T formed visible colonies of cream white, circular- and convex- shaped, with undulating edges. The colony size was approximately 0.5–1.5 mm in diameter on 10% LB agar after 48 h of incubation at 55°C. Strain I1-1^T grew well at temperatures ranging from 35 to 60°C, and pH between 6 and 8. Optimal growth condition was around 55°C and pH 7.0. Light microscopic examination revealed that cells of strain I1-1^T were Gram reaction negative rods of 0.4–0.5 µm in diameter and 0.8–2.0 µm in length. Cells were nonspore-forming and motile by means of single polar flagellum. Poly-β-hydroxybutyrate granules were present. Strain I1-1^T did not grow after 120 h of incubation at 55°C under anaerobic condition.

Phylogenetic analysis

Comparison of *16S rRNA* gene sequence of strain I1-1^T (GenBank nucleotide sequence accession number is AY845054) with available *16S rRNA* gene sequences in

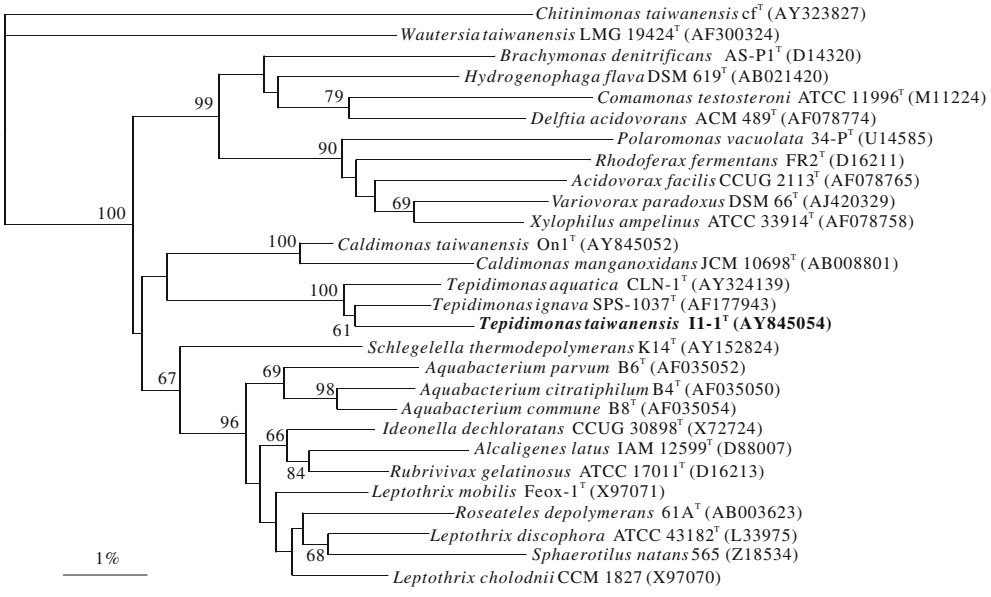

Fig. 1 Neighbor-joining phylogenetic tree of *T. taiwanensis* strain I1-1^T and other closely related bacteria of β-Proteobacteria based on 16S rRNA sequence comparisons. *Scale bar* indicates 1% sequence dissimilarity (one substitution per 100 nt). *Bootstrap values* (%) are indicated at the *branches* from 1,000 replications. Only bootstrap values > 50% are shown. Representative sequences in the dendrogram obtained from GenBank, which were almost complete sequences, were used in the phylogenetic analysis (GenBank accession numbers are shown in *parentheses*). The sequences of *Chitinimonas taiwanensis* cf^T (Chang et al. 2004) and *Wautersia taiwanensis* LMG 19424^T (Chen et al. 2001) were used as the outgroup

Table 1 Fatty acid compositions

Compound	*T. ignava* SPS-1037^T	*T. aquatica* CLN-1^T	*T. taiwanensis* I-1^T
15:1 ω6c	1.8	1.0	1.2
15:0	1.1		
Summed feature 3[a]	20.0	20.4	26.3
16:0	32.8	42.4	40.9
17:0 cyclo	13.8	22.0	5.0
17:0	13.5	5.1	8.2
18:1 ω7c	10.1		13.4
18:0	2.4	4.5	1.9
Unknown	2.1	1.0	1.5
19:0 cyclo ω8c	1.0		

Values for fatty acid present at level of less than 1% in the strain are not given. All strains were grown at 55°C for 48 h on TSA medium, and then the fatty acid composition was analyzed
[a]Summed feature 3 comprises 16:1 ω7c or 15:0 iso 2OH or both

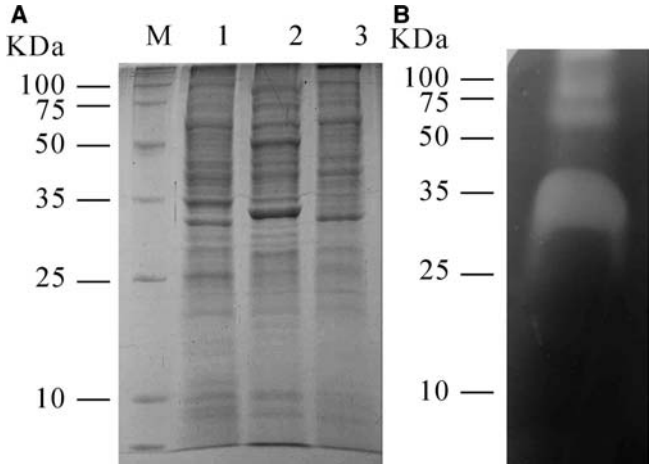

Fig. 2 **a** Electrophoretic protein patterns of *T. ignava* strain SPS-1037^T (*lane 1*) and *T. aquatica* strain CLN-1^T (*lane 2*) and *T. taiwanensis* strain I1-1^T (*lane 3*). Molecular weight standards (*lane M*) are shown at *left*. **b** Zymogram of protease activity in extracellular proteins from *T. taiwanensis* strain I1-1^T. The *numbers in the left* indicate the standard molecular weight

public databases (GenBank and Ribosomal Database Project II) revealed that strain I1-1^T belonged to the *Burkholderiales* group of the β-Proteobacteria. The highest similarity values were obtained with the genera *Tepidimonas*, *Schlegelella*, *Leptothrix*, *Brachymonas*, *Hydrogenophaga* and *Caldimonas*. The 16S rDNA sequence of strain I1-1^T clustered together with *Tepidimonas ignava* strain SPS-1037^T (97.2% similarity) (Moreira et al. 2000) and *Tepidimonas aquatica* strain CLN-1^T (96.8% similarity) (Freitas et al. 2003) (Fig. 1). The similarity level with other bacterial species belonging to the *Burkholderiales* group of the β-Proteobacteria was less than 96%. Subsequently, whole-genome DNA–DNA hybridization experiment was preformed between strain I1-1^T and the type strains of its nearest phylogenetic neighbors, *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T. The binding levels of strain I1-1^T towards *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T were 38.3 and 40.8%, respectively. The guanine-plus-cytosine (G+C) content of strain I1-1^T DNA was 68.1 mol%.

Fatty acid composition

The major cellular fatty acid composition of strain I1-1^T was 16:0, 18:1 ω7c, and summed feature 3 (16:1 ω7c or 15:0 iso 2OH or both). Fatty acid profiles of strain I1-1^T and related species from the genus *Tepidimonas* that grew under the same conditions (55°C for 48 h on TSA medium) were compared using unweighted arithmetic average clustering algorithm. Strain I1-1^T had a fatty acid profile similar to *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T with Euclidian distance of 14.9 and 22.8, respectively. The fatty acids of all strains were qualitatively similar, but the relative proportions of the fatty acids were different (Table 1). The fatty acid identified as 17:0 cyclo, for example, is present in high levels in *T. ignava* and *T. aquatica*, but in lesser amounts in I1-1^T.

SDS-PAGE of whole-cell protein profiles

The whole-cell protein profile of strain I1-1^T was compared with that of *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T. The protein profiles (Fig. 2a) were numerically analyzed to calculate the similarity level between the strains. Strain I1-1^T had a high similarity with *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T, with a similarity value of 92.1 and 89.8%, respectively. Further, a zymogram analysis of strain I1-1^T revealed the presence of protease activity near the molecular weights of ca. 32, 60, and 75 kDa (Fig. 2b).

Biochemical analysis

Strain I1-1^T showed nitrate reduction, gelatin hydrolysis, esculin hydrolysis, oxidase, alkaline phosphatase, C4 esterase, C8 lipase, C14 lipase, leucine arylamidase, valine arylamidase, and naphthol-AS-BI-phosphohydrolase; it showed assimilation of glucose, malate, citrate, acetic acid, α-keto butyric acid, formic acid, L-ornithine, α-keto valeric acid, D,L-lactate, L-proline, L-pyroglutamic acid, propionic acid, α-hydroxybutyric acid, β-methyl D-glucoside, α-keto glutaric acid, L-phenylalanine, 2-amino ethanol, D-glucosaminic acid, L-aspartic acid, L-threonine, glycyl-L-glutamic acid, γ-amino butyric acid, and urocanic acid.

The following biochemical characteristics were negative: catalase, indole production, glucose fermentation, arginine dihydrolase, urease, β-galactosidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase; it showed assimilation of arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine,

Table 2 Differential phenotypic and biochemical characteristics of *T. taiwanensis* and other *Tepidimonas* species

	T. ignava SPS-1037^T	*T. aquatica* CLN-1^T	*T. taiwanensis* 1-1^T
Catalase	+	+	–
Oxidase	+	+	W
Urease	+	–	–
C14 lipase	–	–	+
Cystine arylamidase	+	+	–
Reduction of nitrate	–	+	+
Hydrolysis			
Gelatin	–	–	+
Casein	–	–	+
Starch	–	–	+
Tween 40	–	+	–
Tween 80	–	+	–
Assimilation			
Glucose	–	–	+
Malate	+	–	+
Glycerol	+	–	–
L-Phenylalanine	–	+	+
L-Ornithine	+	–	+
α-Keto glutaric acid	+	–	+
G+C content (mol%)	69.7	68.6	68.1

The data of *T. ignava* SPS-1037^T and *T. aquatica* CLN-1^T were obtained from Moreira et al. (2000) and Freitas et al. (2003)

+ Positive result or growth, *w* weakly positive, - negative result or no growth

Alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase activity were present in all organisms. All strains assimilated proline, acetate, L-aspartate, and lactate. Trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, β-galactosidase, α-galactosidase, α-glucosidase, β-glucosidase, urease and indole were negative in all organisms. None of the strains assimilated arabinose, cellobiose, D-fructose, L-fucose, D-galactose, lactose, maltose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, sucrose, D-trehalose, adonitol, D-arabitol, *i*-erythritol, D-mannitol, *myo*-inositol, D-sorbitol, xylitol, *N*-acetyl-glucosamine, L-histidine, L-serine, or formate

maltose, gluconate, caprate, adipate, phenyl acetate, methyl pyruvate, 2,3-butanediol, D,L-α-glycerol phosphate, glycerol, succinic acid, *i*-erythritol, L-histidine, D-melibiose, ρ-hydroxy phenylacetic acid, turanose, itaconic acid, bromo succinic acid, α-cyclodextrin, D-fructose, *cis*-aconitic acid, L-fucose, succinamic acid, hydroxy L-proline, inosine, glucuronamide, D-galactose, uridine, D- raffinose, alaninamide, thymidine, tween 40, gentiobiose, L-rhamnose, D-galactonic acid lactone, L-alanine, D-alanine, phenyl ethylamine, putrescine, tween 80, D-galacturonic acid, D-sorbitol, *N*-acetyl-D-galactosamine, *myo*-inositol, sucrose, malonic acid, L-alanyl-glycine, D-serine, adonitol, *N*-acetyl-D-glucosamine, α-D-lactose, D-trehalose, lactulose, D-glucuronic acid, L-serine, xylitol, D-saccharic acid, L-glutamic acid, arabitol, mono-methyl succinate, glucose-6-phosphate, glucose-1-phosphate, γ-hydroxybutyric acid, L-leucine, cellobiose, glycogen, quinic acid, L-asparagine, D,L-carnitine, sebacic acid, dextrin, glycyl-L-aspartic acid, β-hydroxybutyric acid and psicose.

Strain I1-1^T was resistant to rifampin, but was susceptible to ampicillin, cefotaxime, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, streptomycin, and tetracycline.

Table 2 indicates the phenotypic and biochemical characteristics between strain I1-1^T, *T. ignava* strain SPS-1037^T, and *T. aquatica* strain CLN-1^T. Strain I1-1^T can be clearly distinguished from *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T by the assimilation of glucose; the hydrolysis of gelatin, casein, and starch; as well as C14 lipase activity.

In the present study, strain I1-1^T was readily distinguished from its nearest phylogenetic neighbors *T. ignava* strain SPS-1037^T and *T. aquatica* strain CLN-1^T by fatty acid compositions (Table 1), whole-cell protein profiles (Fig. 2a), phenotypic and biochemical characteristics (Table 2), and DNA–DNA reassociation results. Therefore, based on phenotypical and phylogenetic criteria, we are of the opinion that the strain I1-1^T isolated from the hot spring merits assignment to a new species within the genus *Tepidimonas* for which the name *Tepidimonas taiwanensis* sp. nov. is proposed.

Description of *Tepidimonas taiwanensis* sp. nov.

T. taiwanensis (tai.wan.en'sis N.L. fem. adj. Taiwanensis, of Taiwan, where the type strain was isolated); cells are Gram reaction negative, rod-shaped, 0.4–0.5 μm in diameter, and 0.8–2.0 μm in length; occur singly. Motile with a single polar flagellum; Poly-β-hydroxybutyrate granules are stored as reserve material; growth is evident at temperature from 35 to 60°C, the optimum being 55°C, and pH from 6.0 to 8.0 with optimum growth at pH 7.0; positive for nitrate reduction, gelatin hydrolysis, esculin hydrolysis, and oxidase activity; glucose, malate, and citrate are utilized; negative for catalase, indole production, glucose fermentation, arginine dihydrolase, urease, and β-galactosidase; arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, and phenyl acetate are not utilized; the major fatty acid components are 16:0, 18:1 ω7c, and summed feature 3 (16:1 ω7c or 15:0 iso 2OH or both); the G+C content of its DNA is 68.1 mol%.

The type strain, I1-1^T, was isolated from the waters of Sih-Chong-Si hot spring, located in Pingtung county of southern Taiwan. The type strain has been deposited at the BCRC (Bioresource Collection and Research Center, Food Industry Research and Development Institute, PO Box 246, Hsinchu, Taiwan, 30099) as BCRC 17406^T and in BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium) as LMG 22826^T.

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